SUMMARY

1. A micro-organism identified as a species of *Pseudomonas* forms adaptive enzymes which oxidize a large number of polyols. The properties and specificities of the enzymes have been studied in cell-free extracts. They are diphosphopyridine nucleotide-linked dehydrogenases converting polyols into ketoses.

2. Sorbitol and dulcitol induce the formation of galactitol dehydrogenase, which oxidizes dulcitol to D-tagatose and accounts for the oxidation of other fully hydroxylated polyols containing an L-*threo* configuration adjacent to a primary alcohol group.

3. Dulcitol also induces the formation of a less stable enzyme (D-iditol dehydrogenase) which accounts for the oxidation of certain fully hydroxylated polyols containing a D-*threo* configuration adjacent to a primary alcohol group.

4. A labile mannitol dehydrogenase occurs in the extracts of cells grown in media supplemented with sorbitol.

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REFERENCES

Block, R. J. (1950). *Analyt. Chem.* **22**, 1327.
Carr, C. J. & Krantz, J. C. (1934). *J. biol. Chem.* **107**, 371.
Dixon, M. (1953). *Biochem. J.* **54**, 437.
Edson, N. L. (1936). *Biochem. J.* **30**, 1862.
Edson, N. L. (1940). *N.Z. med. J.* **39**, 39.
Edson, N. L. (1953). *Aust. N.Z. Ass. Adv. Sci., Rep. 29th Meeting, Sydney*, 1952, **29**, 281.
Embden, G. & Griesbach, W. (1914). *Hoppe-Seyl. Z.* **91**, 251.
Futterman, S. & Roe, J. H. (1955). *J. biol. Chem.* **215**, 257.
Gätzi, K. & Reichstein, T. (1938). *Helv. chim. acta*, **21**, 914.
Glatthaar, C. & Reichstein, T. (1938). *Helv. chim. acta*, **21**, 3.
Hann, R. M. & Hudson, C. S. (1945). *J. Amer. chem. Soc.* **67**, 602.
Hermann, S. & Neuschul, P. (1931). *Biochem. Z.* **233**, 129.
Hough, L. (1950). *Nature, Lond.*, **165**, 400.
Hudson, C. S., Hartley, O. & Purves, C. B. (1934). *J. Amer. chem. Soc.* **56**, 1248.
Isherwood, F. A. & Jermyn, M. A. (1951). *Biochem. J.* **48**, 515.
Kenner, J. & Richards, G. N. (1954). *J. chem. Soc.* p. 1784.
Klevstrand, R. & Nordall, A. (1950). *Acta chem. scand.* **4**, 1320.
Kristensen, M. (1943). *Acta path. microbiol. scand.* **20**, 530.
Levene, P. A. & Jacob, W. A. (1910). *Ber. dtsch. chem. Ges.* **43**, 3141.
Levene, P. A. & La Forge, F. B. (1915). *J. biol. Chem.* **20**, 429.
Lobry de Bruyn, C. A. & Alberda van Ekenstein, W. (1899). *Rec. Trav. chim. Pays-Bas*, **18**, 150.
McCorkindale, J. & Edson, N. L. (1954). *Biochem. J.* **57**, 518.
Meyer, A. S. & Reichstein, T. (1946). *Helv. chim. acta*, **29**, 152.
Monod, J. & Cohn, M. (1952). *Advanc. Enzymol.* **13**, 67.
Ömer, Ö. (1940). *Bull. Fac. Med. Istanbul*, **3**, 1675.
Pizzarello, R. A. & Freudenberg, W. (1939). *J. Amer. chem. Soc.* **61**, 611.
Reichstein, T. & Bosshard, W. (1934). *Helv. chim. acta*, **17**, 753.
Richtmyer, N. K., Stewart, L. C. & Hudson, C. S. (1950). *J. Amer. chem. Soc.* **72**, 4934.
Sebek, O. K. & Randles, C. I. (1952). *J. Bact.* **63**, 693.
Shockley, T. E. & Randles, C. I. (1954). *Proc. Soc. Amer. Bacteriol., 54th General Meeting*, p. 113.
Simon, F. (1947). *Arch. Biochem.* **14**, 39.
Smith, L. (1955). *J. biol. Chem.* **215**, 833.
Smith, W. W., Finkelstein, N. & Smith, H. W. (1940). *J. biol. Chem.* **135**, 231.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Stadtman, E. R., Novelli, G. D. & Lipmann, F. (1951). *J. biol. Chem.* **191**, 365.
Stanier, R. Y., Gunsalus, I. C. & Gunsalus, C. F. (1953). *J. Bact.* **66**, 543.
Teague, O. & Morishimu, K. (1920). *J. infect. Dis.* **26**, 52.
Tobie, W. C. (1945). *J. Bact.* **49**, 459.
Todd, C. M. (1954). *Aust. J. exp. biol. med. Sci.* **32**, 827.
Usami, S. (1942). *J. Fac. Sci. Hokkaido Univ.* Ser. V, **5**, 77.

A New Method for the Isolation of Ribonucleic Acids from Mammalian Tissues

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The isolation of ribonucleic acids (RNA's) from mammalian tissues has been the subject of several procedures (cf. Magasanik, 1955) and none has been entirely satisfactory. The method described by Kay & Dounce (1953) involved separation of the nuclei, and an adjustment of the pH to 4·5 before two treatments with sodium dodecyl sulphate. Grinnan & Mosher (1951) also found it essential to remove cell nuclei. They used guanidine hydrochloride to denature the protein, but it was necessary to heat the preparation at 90–100° and finally to treat with chloroform and butanol to remove the protein. Volkin & Carter (1951) also used guanidine hydrochloride and kept the temperature at 40°, but

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the yield in this case was only 20–30 % of the total tissue RNA.

Westphal, Lüderitz & Bister (1952) were able to isolate pyrogenic polysaccharides from bacteria by treatment with phenol and water. When the extraction was carried out at 3–5° a glycoprotein was found in the aqueous phase, and extraction at 68° caused the protein moiety to split off and resulted in polysaccharides and RNA's being found in the aqueous phase. It seemed possible that this method could be applied to the extraction of RNA's from mammalian tissues. A series of experiments on the extraction of rat liver at 68° showed that it was possible to obtain RNA's by this method, but further investigations have shown that better results could be obtained by performing the extraction at room temperature. After centrifuging, the separation appeared as shown in Fig. 1. All the deoxyribonucleic acid (DNA) was insoluble, but the problem remained to separate RNA from polysaccharides present in the liver. Westphal *et al.* (1952) devised a somewhat lengthy precipitation and ultracentrifuging procedure for the separation, and some degree of fractionation could be achieved by similar methods in extracts of rat liver. Experience with RNA in two-phase systems suggested that these might be applicable, and it was possible to separate rat-liver RNA completely from the polysaccharide by extraction with 2-methoxyethanol from potassium phosphate solutions. The RNA was all in the organic layer, but the polysaccharide was partially insoluble and partially soluble in the aqueous layer.

EXPERIMENTAL AND RESULTS

Preparation and analysis of ribonucleic acids

Rat liver. Rats were killed by fracturing the neck, and the livers were quickly cut out and immediately dropped on to solid CO_2. The pooled livers were thawed and homogenized in water in a Waring Blendor for about 60 sec. (Smaller quantities could be satisfactorily treated in ground-glass or polythene homogenizers of the Potter–Elvehjem type.) The homogenate was poured through a large Büchner funnel with fine pores, to remove fibrous tissue, and the mixture was stirred while an equal volume of 90 % (w/v) phenol was added quickly. Stirring was then continued for 1 hr., but very small quantities could be treated equally well by vigorous shaking for the same period. The mixture could be centrifuged immediately or allowed to remain overnight, after which centrifuging was carried out in an International centrifuge at 1750 rev./min. for 1 hr. at 0°. The cloudy aqueous layer was removed by suction and the residue washed three times with water. The aqueous layer was separated each time by centrifuging for 45 min. and removed by suction. The combined aqueous extracts were made up to 2 % with respect to potassium acetate, and the RNA was precipitated by addition of 2 vol. of ethanol.

The precipitate was separated by centrifuging (1750 rev./min. for 20 min.), washed once with ethanol–water (3:1), redeposited by centrifuging and the insoluble portion dissolved in 25–100 ml. of water (depending upon the amount present). Ethanol was removed either in a rotary evaporator of the type described by Lea, Hannan & Rhodes (1951) at 20–25°, or by extracting three times with an equal volume of ether, when any emulsion which formed was usually dispersed easily by shaking vigorously, after removing the aqueous layer, or by centrifuging. Ether was then removed by blowing nitrogen through the aqueous layer.

The clear aqueous solution was mixed with an equal volume of 2·5 M-K_2HPO_4, 0·05 vol. of 33·3 % H_3PO_4 (prepared by mixing 1 vol. of AnalaR H_3PO_4, sp.gr. 1·73, with 2 vol. of water) and 1 vol. of 2-methoxyethanol (redistilled, b.p. 122–124) (upper phase:lower phase, 5:1). The lower layer together with any insoluble material was separated either by standing or by centrifuging, and washed once with 10–20 ml. of the top layer from a mixture of 2-methoxyethanol–water–2·5 M-K_2HPO_4–33·3 % H_3PO_4 (1:1:1:0·05, by vol.). The combined top layers contained all the RNA and any insoluble matter could be removed by centrifuging in polythene tubes in a Servall high-speed centrifuge at 10000 *g* for 1 hr. The clear supernatants were combined, a few drops of toluene were added and the whole was dialysed against water (2 l.), which was changed at least four times.

The first diffusate gave a positive ribose test, indicating that nucleotides of small molecular weight were present in the extract. (An alternative method was to dialyse the methoxyethanol layer directly and then to clarify this by centrifuging in the Spinco model L centrifuge at 21000 rev./min. for 1 hr.)

The contents of the dialysis bag were centrifuged if necessary, made up to 2 % with respect to potassium acetate and then precipitated by addition of 2 vol. of ethanol. The precipitate was collected by centrifuging, washed two or three times with ethanol–water (3:1) and then dried in a vacuum desiccator over P_2O_5.

The product was a hard, friable, resin-like mass, which contained about 12 % of moisture and was completely soluble in water. The yield was usually of the order 450–650 mg./100 g. of rat liver, and $E^{1\%}_{1\,cm.}$ (260 mμ.) in water was about 200 (ϵ_P, 8200). Low $E^{1\%}_{1\,cm.}$ values showed that all polysaccharides had not been removed, and in these cases

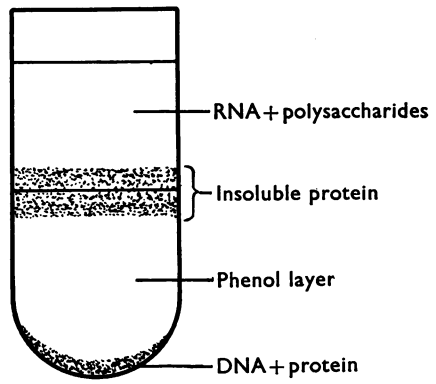

Fig. 1. Appearance of the separation of the extract of rat liver after centrifuging.

Table 1. *Analyses and base ratios of RNA's*

Base ratios are expressed on a molar basis with respect to adenine.

Strain of rat	Guanine	Adenine	Cytosine	Uracil	N (%)	P (%)	N/P	N/P (calc.)
				(*a*) Rat liver				
August	1·79	1·0	1·60	0·87	11·2	6·67	1·67	1·75
Wistar	1·80	1·0	1·54	0·84	11·65	6·75	1·72	1·75
Marshall	1·81	1·0	1·63	0·91	11·85	6·5	1·82	1·74
				(*b*) Calf pancreas				
—	3·13	1·0	1·06	0·39	11·8	5·85	2·01	1·99

the product was dissolved in water, re-extracted with potassium phosphate and 2-methoxyethanol and the RNA isolated as described previously.

Paper chromatography of the hydrolytic products from heating the RNA obtained before extraction by 2-methoxyethanol always showed a glucose spot in addition to a ribose spot, and glucose was also present in products with low $E^{1\%}_{1\,cm.}$ values. RNA purified by 2-methoxyethanol showed only a ribose spot by the same treatment.

Base ratios were determined in triplicate by hydrolysing the RNA (1·5 mg.) in N-HCl (0·05 ml.) for 1 hr. at 100°, and 0·01 ml. of the resulting clear solution was used for each determination. The solvent system (methanol–ethanol–water–11·6 N-HCl, 50:25:19:6, by vol.) used for separating the products has been described previously (Kirby, 1955). A further 0·01 ml. of this solution was used for detecting sugars by paper chromatography.

Hydrolysis of RNA's with 5·8 N-HCl for 16 hr. at 100° showed only very faint traces of amino acids by paper chromatography.

Analyses and base ratios of rat-liver RNA's are shown in Table 1 (*a*). Nitrogen and phosphorus values were obtained from material containing about 12% of moisture.

The RNA's from rats of the Marshall strain were obtained by treatment with phenol and water at 68°, the others were isolated at room temperature. The ratio N/P (calc.) is the figure obtained from the base ratios when 1 atom of P/residue of base is assumed.

Other rat tissues. The same method has been applied successfully to rat kidney, spleen, a transplantable sarcoma and especially to many liver tumours induced by 4-dimethylaminoazobenzene. The results of these will be reported later.

Calf pancreas. The calf pancreas was kept in a Dewar flask with solid CO_2 for 1 week before being used. Calf pancreas (100 g.), freed from connective tissue, produced 279 mg. of RNA; $E^{1\%}_{1\,cm.}$, 199 ($\lambda_{max.}$, 256 mμ.). Analyses and base ratios are shown in Table 1 (*b*).

It will be noticed that a very low pyrimidine content was obtained, which may have been the result of keeping the material before use, but the $E^{1\%}_{1\,cm.}$ seemed to indicate that no extensive degradation had occurred.

Rous tumour. Previously frozen tumour (80 g.) was treated as described above, and RNA together with hyaluronic acid appeared in the aqueous layer. However, the hyaluronic acid was extracted with the RNA into the organic layer of the 2-methoxyethanol–K_2HPO_4 mixture. No separation was achieved when 4% of sodium borate was used in the place of the water in the mixture (4%, w/v, sodium borate–2·5 M-K_2HPO_4–2-methoxyethanol–33% H_3PO_4; 1:1:1:0·05, by vol.), a result in agreement with the proposed structure for hyaluronic acid which contains no *cis*-diol groupings (Meyer, Fellig & Fischer, 1951; Jeanloz & Forchielli, 1951). Since Beale, Harris & Roe (1952) have shown that Rous-tumour RNA could be satisfactorily separated from hyaluronic acid by the use of hyaluronidase the investigation was not continued.

Inhibition of pancreatic ribonuclease by treatment with phenol and water

Pancreatic ribonuclease (3 mg.) was dissolved in water (10 ml.) and 5 ml. of this solution was mixed with 5 ml. of 90% (w/v) phenol, and the mixture shaken for 1 hr. The phenol layer was separated and the aqueous layer extracted five times with ether. The ether was removed from the water by bubbling nitrogen through the solution, which was then made up to 5 ml. again.

Volumes of the treated and untreated ribonuclease solutions (0·5 ml. of each) were added to 2 ml. portions of a solution of purified yeast ribonucleic acid (60 mg.) dissolved in 5 ml. of 0·2 M sodium acetate. After remaining for 18 hr. at 20°, 0·01 ml. of each solution was spotted on to paper and chromatographed with the solvent system methanol–ethanol–water–11·6 N-HCl (50:25:19:6, by vol.). (See Kirby, 1955.)

Some fluorescent material had moved a few centimetres from the origin in each case, but uridylic and cytidylic acids could be detected only in the system with yeast RNA and untreated ribonuclease. The two nucleotide spots observed had the correct R_F values and were eluted with 0·2 M sodium acetate. The ultraviolet-absorption curves were characteristic of each component.

DISCUSSION

The present method has a number of advantages over those previously published. The whole preparation is carried out at pH 6·0–7·5. Pancreatic ribonuclease has been shown to be either denatured or extracted by phenol, and since this is a relatively stable enzyme it is reasonable to assume that most other nucleases were similarly inactivated. The main advantage is that up to the moment no case has been encountered where the DNA has been solubilized. It is not therefore necessary to separate the nuclei before the preparation, and the method should be eminently suitable for the preparation of RNA from nuclei. So far proteins have caused no difficulties, and all RNA's prepared by this method have been virtually protein free. The main difficulty

(which has been encountered with many RNA's) was the clean separation from polysaccharides, and, although it is possible to reduce glycogen contamination of liver preparations by starving the animals for 24 hr. before the experiments, the RNA's could be separated by the 2-methoxyethanol technique without either perfusing the liver or starving the animals. This particular two-phase system may not always be successful, and an example of this is given in the attempted isolation of RNA from Rous tumour, but there is little doubt that other effective systems could be found. The extraction of hyaluronic acid into the organic layer suggests that similar two-phase systems may be of value in fractionating polysaccharides.

The separation of RNA's from mammalian tissues by treatment with phenol indicates that in the main these poly-acids cannot be attached to the proteins electrostatically. It seems reasonable to assume that hydrogen bonds exist between the bases of the RNA's and the proteins concerned, and that these bonds are broken by the phenol. No doubt the solvent properties of phenol are important in the separation, as a considerable amount of protein can be precipitated from the clarified phenol layer by addition of sodium acetate and ethanol.

SUMMARY

1. Ribonucleic acids have been prepared from mammalian tissues by extraction with phenol and water at room temperature.

2. Deoxyribonucleic acids remain completely insoluble under the conditions used.

3. Ribonucleic acids have been separated from polysaccharides by extraction with 2-methoxyethanol from potassium phosphate solution.

4. Pancreatic ribonuclease was inactivated by the same phenol treatment.

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REFERENCES

Beale, R. N., Harris, R. J. C. & Roe, E. F. M. (1952). *J. chem. Soc.* p. 1034.

Grinnan, E. L. & Mosher, W. A. (1951). *J. biol. Chem.* **191**, 719.

Jeanloz, R. W. & Forchielli, E. (1951). *J. biol. Chem.* **190**, 537.

Kay, E. R. M. & Dounce, A. L. (1953). *J. Amer. chem. Soc.* **75**, 4041.

Kirby, K. S. (1955). *Biochim. biophys. Acta*, **18**, 575.

Lea, C. H., Hannan, R. S. & Rhodes, D. N. (1951). *Biochim. biophys. Acta*, **7**, 377.

Magasanik, B. (1955). In *The Nucleic Acids*, vol. 1, p. 373. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.

Meyer, K. H., Fellig, J. & Fischer, E. H. (1951). *Helv. chim. acta*, **34**, 939.

Volkin, E. & Carter, C. E. (1951). *J. Amer. chem. Soc.* **73**, 1516.

Westphal, O., Lüderitz, O. & Bister, F. (1952). *Z. Naturf.* B, **7**, 148.

A Windowless Flow-type Geiger Counter for the Assay of Solid Materials Containing Soft β-Emitting Isotopes

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Although gas-counting and scintillation-counting methods are more efficient than solid-sample counting methods for isotopes emitting only soft β-particles, there are many occasions when measurement of the radioactivity of a solid sample, with a suitable Geiger or proportional counter, constitutes a perfectly satisfactory and sufficiently sensitive method of assay. The method also has much to recommend it from the point of view of speed, simplicity, and the ability to recover the sample unchanged after assay.

The thinnest mica windows on Geiger counters commercially available in this country absorb over 50% of the β-particles from ^{35}S or ^{14}C, and do not permit the passage of ^{3}H β-particles at all. By the use of a windowless counter the sensitivity with which ^{14}C or ^{35}S may be detected and measured in solid samples can, therefore, be at least doubled, and the method is theoretically also applicable to the measurement of ^{3}H.

Windowless counters for solid samples may be of two main types: demountable, and continuous gas